Prurigo Nodularis Is Characterized by Systemic and Cutaneous T Helper 22 Immune Polarization

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Prurigo nodularis (PN) is an understudied, chronic inflammatory skin disease that disproportionately affects African Americans and presents with intensely pruritic nodules of unknown etiology. To better characterize the immune dysregulation in PN, PBMCs and skin biopsies were obtained from patients with PN and healthy subjects (majority African American) matched by age, race, and sex. Flow cytometric analysis of functional T-cell response comparing patients with PN with healthy subjects identified increased γδT cells (CD3−CD4+ CD8− γδTCR+) and Vδ2+ γδT enrichment. Activated T cells demonstrated uniquely increased IL-22 cytokine expression in patients with PN compared with healthy controls. CD4+ and CD8+ T cells were identified as the source of increased circulating IL-22. Consistent with these findings, RNA sequencing of lesional PN skin compared with nonlesional PN skin and biopsy site–matched control skin demonstrated robust upregulation of T helper (Th) 22–related genes and signaling networks implicated in impaired epidermal differentiation. Th22–related cytokine upregulation remained significant, with stratifications by race and biopsy site. Importantly, the expression of the IL-22 receptors IL22RA1 and IL22RA2 was significantly elevated in lesional PN skin. These results indicate that both systemic and cutaneous immune responses in patients with PN are skewed toward a Th22/IL-22 profile. PN may benefit from immunomodulatory therapies directed at Th22–mediated inflammation.


INTRODUCTION

Prurigo nodularis (PN) is a chronic inflammatory skin disease characterized by intensely pruritic nodules on the extremities and trunk (Kwatra, 2020). PN disproportionately affects African Americans and is associated with multiple systemic conditions such as cardiovascular disease and type II diabetes (Boozalis et al., 2018; Huang et al., 2020b, 2020c; Whang et al., 2019a, 2019b). Among chronic pruritic dermatoses, PN has the greatest itch intensity and causes the most significant impairment of QOL (Huang et al., 2020b; Kwatra, 2020; Steinke et al., 2018; Williams et al., 2020). Given PN's unknown etiology and the lack of United States Food and Drug Administration–approved treatments, the current therapeutic strategies for PN remain largely ineffective (Huang et al., 2020a; Kwatra, 2020; Williams et al., 2020).

Previous investigations of PN have suggested roles for aberrant keratinocyte (KC) signaling, neuronal dysregulation, and cutaneous inflammation (Haas et al., 2010; Liang et al., 2000; Matsumura et al., 2015; Wong et al., 2020; Zhong et al., 2019). Targeted real-time PCR and immunohistochemical staining have demonstrated mixed evidence of dysregulation of neurotrophic factors and cytokines, with studies suggesting that PN has variable amounts of T helper (Th2, Th17, or Th22 signaling (Bağıcı and Ruzicka, 2018; Beck et al., 2014; Fukushima et al., 2011; Park et al., 2011; Wong et al., 2020). Recent studies of atopic dermatitis (AD), a chronic inflammatory pruritic skin disease that is comorbid in 11–19% of patients with PN, have also revealed significant immune-based variations between racial groups (Boozalis et al., 2018; Iking et al., 2013; Noda et al., 2015;
Figure 1. PN lesional skin is characterized histologically by marked inflammatory cell infiltrate, robust architectural changes, and greater IF signal intensity of IL-1A and SERPINB4. (a) H&E staining of lesional PN, nonlesional PN, and matched healthy control skin tissue biopsies from African American females. (b) Average epidermal thickness as measured at the thickest suprapapillary epidermal plate. (c) Blinded dermatopathologist assessed the degree of inflammation (0 = no, 3 = severe). (d) Blinded dermatopathologists assessed the presence (1) or absence (0) of specific inflammatory cell infiltrate across different skin samples with the average value shown. (e) IF staining of DAPI, IL-1A, and SERPINB4 in lesional PN and matched healthy skin from African American females, with
Sanyal et al., 2019). Notably, African American patients with AD display Th2/Th22 skewing (Sanyal et al., 2019). Such Th2/Th22 cytokine polarization may explain why African American patients with AD experience a more severe AD phenotype with extensor body area involvement and increased papular clinical manifestations (McColl et al., 2021).

IL-22, a member of the IL-20 cytokine subfamily and larger IL-10 cytokine family, is a major proinflammatory cytokine produced by circulating lymphocytes, including Th17 cells, Th22 cells, invariant natural killer (iNK) T cells, and γδ T-cells (Mashiko et al., 2015; Rutz et al., 2014; Zheng and Li, 2018). Elevated serum IL-22 is implicated in numerous chronic inflammatory conditions such as coronary artery disease and type II diabetes, both of which are highly associated comorbidities in patients with PN (Gong et al., 2016; Rutz et al., 2014; Zheng and Li, 2018). A previous study demonstrated

Figure 2. Analyses of DEGs in L PN (n = 13), NL PN (n = 13), and matched healthy skin (n = 13). DEGs are defined as coding genes with a log base 2 Fc value less than -1 or >1 and FDR-adjusted P < 0.05 (-1 > logFC > 1, P < 0.05). (a) Heatmap of all the DEGs by RNA seq in L PN, NL PN, and matched healthy control skin. Red, greater expression; blue, lower expression. (b–d) Volcano plot compared gene expression in (b) L with that in NL PN skin, (c) the gene expression in L with that in healthy skin, and (d) gene expression in NL with that in healthy skin. (e) Venn diagram comparison of DEGs. (f–h) Plot comparison of DEG Fc. (f) L/H/L/NL, (g) NL/H/L/NL, and (h) NL/H/L. DEG, differentially expressed gene; Fc, fold change; FDR, false discovery rate; H, matched healthy control sample; L, lesional; NL, nonlesional; PN, prurigo nodularis; RNA seq, RNA sequencing.

overlapping areas highlighted in gold. *P < 0.05, **P < 0.01, and ***P < 0.001. × 5 Bar = 200 μm; × 10 bar = 100 μm; × 20 bar = 50 μm; × 40 bar = 20 μm; IF bar = 200 μm. E, eosinophil; IF, immunofluorescence; L, lymphocyte; M/H, macrophages/histiocytes; MC, mast cell; N, neutrophil; PC, plasma cell; PML, polymorphonuclear leukocyte; PN, prurigo nodularis.
Figure 3. Cutaneous mRNA analyses indicate Th22 immune polarization. (a) A heatmap of cutaneous mRNA expression of select Th22/IL-22–related genes. Red, greater expression; blue, lower expression. The fold change shown is log base 2 fold change. The P-value shown is a false discovery rate–adjusted P-value. (b) GeneMANIA functional association gene network for Th22-associated genes. The coexpression and physical interaction between the genes are expressed as purple and green lines, respectively. Stronger associations are shown with thicker lines. Gene names shown in red are upregulated, whereas the names in black show no significant difference in expression in PN L skin compared with that in healthy controls. (c) GSVA comparison of immune mediators in L PN (n = 13) with those in NL (n = 13) and healthy (n = 13) skin found that L PN skin is characterized by significantly increased expression of Th17 and Th22 markers, mildly elevated Th1, and no significant difference in the expression of Th2-related genes. *P < 0.05, **P < 0.01, and ***P < 0.001. GSVA, gene set variation analysis; H, matched healthy control samples; L, lesional; NL, nonlesional; PN, prurigo nodularis; Th, T helper.
a correlation of IL-22 plasma levels with disease severity in patients with psoriasis, a condition also known to be associated with coronary artery disease and type II diabetes (Daudén et al., 2013; Lønnberg et al., 2016; Wolk et al., 2006). In the epidermis, elevated levels of IL-22 promote KC hyperplasia and acanthosis as well as function synergistically with IL-17 to upregulate antimicrobial peptides (Boniface et al., 2005; Sonnenberg et al., 2011; Zheng et al., 2007). Because PN is characterized both clinically and histopathologically by KC hyperplasia and impaired epidermal differentiation, we hypothesized that PN would display Th22 polarization.

Improved molecular immune characterization has led to major breakthroughs in the management of chronic inflammatory diseases, such as AD, psoriasis, and rheumatoid arthritis (Florian et al., 2020; Hawkes et al., 2017; O’Dell, 2004; Thijs et al., 2017). When applied to PN, a similar strategy may better identify targeted therapies and guide the use of existing or emerging treatments. However, no study to date has performed circulating blood immunophenotyping and global cutaneous gene transcriptome analysis in patients with PN. Therefore, to better characterize the immune response in patients with PN, we performed flow cytometry of PBMCs along with RNA sequencing and targeted immunohistochemistry of lesional PN, performing molecular characterization of cutaneous psoriasis.
nonpruritic nonlesional PN, and healthy matched skin samples from a cohort of predominantly African American patients (Supplementary Tables S1 and S2). We observed that the systemic and cutaneous immune responses in patients with PN are skewed toward a Th22/IL-22 profile and that the degree of cutaneous upregulation correlates with itch severity.
RESULTS
Lesional PN skin is characterized histologically by marked inflammatory cell infiltrate and significant architectural changes
Histologic evaluation of lesional PN skin demonstrated significantly increased epidermal thickness as well as hyperkeratosis, hypergranulosis, acanthosis, spongiosis, increased vascularity, and mild dermal fibrosis (Figure 1a–c) compared with nonlesional PN and healthy skin samples. Lesional PN skin displayed significantly greater inflammation with increased neutrophil, eosinophil, mast cell, and plasma cell infiltrates (Supplementary Figure S1) compared with matched healthy skin (Figure 1d). Only mast cell infiltration was appreciably greater in lesional PN than in nonlesional PN skin (Figure 1d).

Lesional PN skin demonstrates a distinct pattern of mRNA expression from that of nonlesional PN and healthy skin
Skin transcriptome profiles were performed in pruritic lesional PN skin, nonpruritic nonlesional PN skin, and matched healthy control skin (Figure 2a). We identified all differentially expressed genes, defined as coding genes with a log2 fold change greater than 1 or less than −1 and a false discovery rate–adjusted P < 0.05 (Figure 2b–d). Lesional PN skin demonstrated a distinct pattern of mRNA expression from that of nonlesional PN and healthy skin (Figure 2e). A significant correlation in mRNA expression differences was observed between lesional PN and nonlesional PN skin as well as between lesional PN and healthy skin (Figure 2f). Few significant differences in mRNA expression were observed between nonlesional PN and matched healthy skin (Figure 2e–h). The full lists of differentially expressed genes for all comparisons are provided in Supplementary Tables S4–S8.

Lesional PN skin shows Th17/Th22 skewing
mRNA sequencing revealed that lesional PN samples expressed significantly increased levels of Th17/Th22-associated S100 genes (S100A7/A8/A9/A12), LOR, and IL-36G compared with nonlesional PN and healthy control skin samples. We also observed significantly increased expression of Th22/IL-22–associated genes, including SERPINB4; CALML5; CCL7; CXCL5; and IL-1β, as well as IL-22 receptors IL22RA1 and IL22RA2 (Figure 3a and b and Supplementary Figure S2). We subsequently observed significantly increased expression of affected downstream genes such as IL-1β–induced keratin genes, K6 and K17 as well as SERPINB4-induced IL1A. Increased SERPINB4 and IL-1α in lesional PN compared with matched healthy skin were also observed with immunofluorescence (Figure 1e). Increased expression of Th17/IL-17–induced genes was additionally observed. Th2-associated signal transducer and activator of transcription 4 gene, STAT4 and CCL13 expressions were significantly decreased, whereas no significant differences in expression was observed for several Th2-related genes, including IL4, IL13, CCL17, CCL12, CCR5 and signal transducer and activator of transcription 6 gene, STAT6; CCL17 and/or CCL22; and CCR5 (P > 0.05) (Supplementary Figure S2).

Gene set variation analyses (GSVAs) were performed to compare the expression of Th1, Th2, Th17, and Th22 markers. Lesional PN skin demonstrated robust increased Th22-related gene expression. In addition, we observed a significantly increased Th17 marker expression, a mild yet statistically significant increase in Th1 marker expression, and no significant differences in Th2-related genes (Figure 3c). To correlate the clinical findings with the degree of immune dysregulation, we compared itch severity with GSVA for Th1−, Th2−, Th17−, and Th22-related genes in pruritic lesional PN skin and biopsy-matched nonpruritic healthy control skin (Supplementary Figure S3). Among these gene families, itch severity most strongly correlated with the degree of Th22-related gene dysregulation (R = 0.89, P < 0.001). GSVA by race subgroups revealed significant Th22 and Th17 skewing to be present in both European and African Americans (Supplementary Figure S4). We only observed a trend toward greater Th2 skewing in Europeans, with decreased expression of Th2 cytokines IL-4, IL-5, IL-13, and IL-31 in African Americans compared with that in Europeans (Supplementary Table S7). GSVA by biopsy site subgroups (arms, legs, back) revealed only Th22 skewing to be consistent across all sites (Supplementary Figure S5). Interestingly, no single anatomic site was clearly the most pruritic across the patients.

Vδ2+ γδ T cells and inNK T cells are enriched in PN
To immunophenotype T cells and their cytokine expression in PN, PBMCs from patients with PN and healthy controls were stimulated with a pan-T-cell cocktail containing phorbol 12-myristate 13-acetate and ionomycin for 4 hours at 37 °C. The stimulated cells were evaluated for T-cell responses, and their cytokine profiles were evaluated by flow cytometry. Cells were stained with surface T-cell markers, including CD3, CD4, CD8, γδ TCR, and CD56, along with intracellular cytokine markers, including IL-22, TNF, IL-4, TGF-β, IFN-γ, IL-17, IL-10, and IL-13. T-cell populations were assayed (the flow cytometry gating strategy is shown in the online Supplementary Materials and Methods and Supplementary Figure S6a–d).

γδ T cells (CD3−/CD4−/CD8−gdTCR+) were increased (Figure 4a and b), and within the γδ T cells, Vδ2+ γδ T cells were enriched on stimulation in subjects with PN when compared with those in healthy controls (Figure 4c and d). Interestingly, iNK T cells (CD3+/CD4−/CD8−/CD56+) were also significantly increased in PN (Figure 4e and f). The absolute numbers for cell populations of γδ T cells and iNK T cells are shown in online Supplementary Materials and Methods and in Supplementary Figure S7a–c. There were reduced CD4+ T cells in PN samples compared with controls (Figure 4g). The CD4 (CD3+CD4+CD8−) to CD8 (CD3−CD8+) ratio and its corresponding absolute numbers are presented in Figure 4d–g. Naive CD8 T-cells (CD3+CD8−CD45RA+) were significantly increased in subjects with PN (Figure 4h and i). There were no differences in CD8+ memory T cells (CD3+CD8−CD45RO−) or in the expression of CD8+ T cells in patients with PN when compared with healthy controls (Figure 4g–i).

Activated T cells show Th22 skewing in PN
To determine the cytokine profile of T cells in PN, stimulated PBMCs were stained for intracellular cytokine markers, including IL-22, TNF, IL-4, TGF-β, IFN-γ, IL-17, IL-10, and
IL-13. T-cell populations and their cytokine profile were assayed. Activated T cells showed increased IL-22 cytokine expression in PN compared with healthy controls (Figure 5a and b). CD4+ and CD8+ T-cell populations were identified as the source of increased circulating IL-22 (Figure 5c). The expression of TNF was marginally increased in subjects with PN (Figure 5d and e). Among T-cell populations, iNKT T cells showed increased expression of TNF cytokine when compared with those in the healthy controls (Figure 5f). There was no difference in the expression of IL-4 cytokine between patients with PN and the healthy controls (Figure 5g and h). However, there was a modest increase in IL-4 cytokine expression observed in iNKT T cells in subjects with PN (Figure 5i). The absolute numbers for cell populations expressing IL-22, TNF, and IL-4 are shown in Supplementary Figure S8a–c. Interestingly, TGFβ expression in γδ T cells was markedly reduced in subjects with PN (Supplementary Figure S9a). Furthermore, no differences in cytokine expression of IFN-γ, IL-17, IL-10, and IL-13 between subjects with PN and healthy controls were observed on stimulation (Supplementary Figure S9b–e). Interestingly, PBMCs from subjects with PN did not express significant amounts of IL-17. To validate our flow expression of IL-17, PBMCs from patients with bacteremia showed increased IL-17 expression (Supplementary Figure S10), suggesting that there was indeed less IL-17 expression from subjects with PN.

**DISCUSSION**

Our results demonstrate that PN is characterized by distinct circulating and cutaneous Th22 immune dysregulation. We also identified circulating CD4+ and CD8+ T-cell populations as the source of increased IL-22 secretion. Although AD and psoriasis have been grouped into dominant Th2 and Th17 immune phenotypes, respectively, similar analyses have not been explored in PN, a condition with equally severe comorbidities and greater pruritus intensity (Boozalis et al., 2018; Gudjonsson et al., 2010; Huang et al., 2020b; Sanyal et al., 2019; Schedel et al., 2014; Suárez-Fariñas et al., 2013; Whang et al., 2019a; Zhong et al., 2019).

The cytokine IL-22 is important in the modulation of tissue responses during skin inflammation and wound healing. Specifically, IL-22 is known to increase the expression of epidermal differentiation complex related S100 genes (S100A7, S100A9, and S100A12); matrix metalloproteinase 3 gene, MMP3; chemokines (CCL7 and CXCL5); serine proteinase inhibitors (SERPINB1 and SERPINB4); and proinflammatory cytokatories such as IL-1β (Boniface et al., 2005; Eyerich et al., 2009; Zheng and Li, 2018). Activation of these genes drives numerous inflammatory processes such as IL-1β–induced K6 and K17, known to be involved in cutaneous inflammation, wound repair, and KC hyperproliferation, as well as SERPINB4–induced IL-1α, known to increase the expression of epidermal differentiation complex genes, proenzymes, and antimicrobial transcripts (Komine et al., 2001; Mee et al., 2007; Titapwatanakun et al., 2005; Zhang et al., 2019). Lesional skin among patients with PN in our study demonstrated significantly increased mRNA levels of each of these genes. PN’s nodular clinical presentation as well as our histologic findings of epidermal hyperplasia, acanthosis, and significant inflammatory infiltrate in PN lesional skin are highly reflective of Th22/IL-22 immune activation (Boniface et al., 2005; Eyerich et al., 2009; Zheng et al., 2007). In addition, elevated IL-22 has been observed in patients with coronary artery disease and type II diabetes, suggesting a pathophysiologic link between PN and its associated increased risk of metabolic and coronary diseases (Boozalis et al., 2018; Gong et al., 2016; Huang et al., 2020b, 2020c; Whang et al., 2019a, 2019b).

We observed significantly increased Vδ2+ γδ T cells in the PBMCs of patients with PN and found that activated T cells showed Th22 polarization. Previous studies have also observed γδ T cells to be significant sources of IL-22; however, the role of circulating γδ T cells in cutaneous disease pathogenesis remains poorly understood (Ness-Schwickerath and Morita, 2011). In addition, we observed elevated expression of IL-22 receptors IL22RA1 and IL22RA2 in lesional PN skin. Because IL-22R expression is largely confined to epithelial cells, implying tissue specificity, our results suggest that persistently elevated systemic IL-22 drives PN in combination with cutaneous IL-22R upregulation with downstream cutaneous immunomodulation resulting in epidermal differentiation and accompanying severe pruritus (Lou et al., 2017; Wolk et al., 2004).

In addition to elevated Th22 markers in lesional PN skin, GSVAs showed Th17 marker elevation, relatively less upregulation of Th1-associated genes, and no significant change in Th2-associated genes. Of note, elevated Th17 markers in PN skin were observed without corresponding elevations in circulating IL-17 or IFN-γ cytokine expression among PBMCs from patients with PN. Therefore, the observed increase in localized Th17-associated genes in patients with PN may result from shared cutaneous Th17 and Th22 gene signatures (Nograles et al., 2008). Alternatively, increased cutaneous Th17-related markers may reflect localized IL-17–mediated hyperkeratosis as observed in keloid pathogenesis or mast cell–secreted IL-17 as seen in koebnerization among patients with psoriasis (Li and Liu, 2019; Lee et al., 2020). Previous studies have shown that mast cells are among the predominant cell types containing IL-17 in human skin, and consistent with previous studies, we observed notably increased mast cell infiltration in lesional PN compared with those in both nonlesional PN and healthy skin (Liang et al., 1998; Lin et al., 2011). PAR2, which provoked mast cell accumulation in murine models, and IL-1β, known to induce human mast cell degranulation, were also significantly elevated in our lesional PN skin samples (Lin et al., 2011; Liu et al., 2016). In addition, the effects of IL-22 are known to be context dependent (Sonnenberg et al., 2011). IL-22, in combination with IL-17, promotes inflammation, inhibits KC differentiation, and increases KC proliferation (Deng et al., 2016; Sonnenberg et al., 2011). Therefore, the elevated cutaneous Th17-related markers may act as local catalysts to drive principally Th22-mediated inflammation.

Interestingly, our unstratified analyses did not observe significant IL-31 upregulation, a key mediator previously implicated in PN (Mikhak et al., 2019; Sonkoly et al., 2006). Because IL-31 is primarily expressed on nerve tissue in the epidermis and spinal cord, IL-31 expression may be reduced in bulk RNA sequencing techniques such as used in our study (Cevikbas et al., 2014; Feld et al., 2016). However, when stratified by race, we observed increased IL-31 expression in...
Europeans compared with that in African Americans. Indeed, previous studies showing IL-31 upregulation consisted largely of European American patients with PN (Mikhak et al., 2019; Sonkoly et al., 2006). This immune heterogeneity may be particularly important because a recent trial of the IL-31 inhibitor nemolizumab showed significantly reduced pruritus in patients with PN; yet, 97% of the patients were European Americans, highlighting the need for greater diversity in future clinical trials (Ständer et al., 2020).

Studies of patients with AD, widely accepted as Th2 centric, have revealed variation in cytokine predominance between racial groups, including the predominance of a Th2 and Th22 molecular phenotype in African American patients (Brunner and Guttmann-Yassky, 2019; Noda et al., 2015; Nomura et al., 2018; Sanyal et al., 2019). Our results reveal Th22 skewing in both African American and European patients. Given previously observed increased expression of IL-22 in two smaller studies of Asian patients with PN, our findings suggest that Th22-related cytokine upregulation in PN may be independent of race (Park et al., 2011; Wong et al., 2020). In contrast, we observed significantly increased expression of Th2 cytokines, including IL-4, IL-5, and IL-13, as well as greater Th2 activation by GSVA among European patients but not among African American patients. Fukushi et al. (2011) previously showed Th2 as a principal driver of PN in a Japanese cohort (Fukushi et al., 2011). Similarly, case reports of PN responding to dupilumab, a mAb targeting IL-4Rα, involved predominantly European or Hispanic patients (Calugareanu et al., 2019; Rambhia and Levitt, 2019; Tanis et al., 2019). We observed cutaneous upregulation of IL-4Rα, without significant concomitant upregulation in cutaneous Th2-associated genes and minimal circulating IL-4 upregulation. It is therefore possible that dupilumab may be most effective in certain endotypes of patients with PN with associated atopy, that IL-4Rα may also be involved in the signaling of other pruritogens in PN, or that our observed nonsignificant changes in Th2 expression in our African American cohort may represent a characteristic molecular phenotype of this specific population. In addition, although previous studies have shown that cutaneous inflammatory activation varies anatomically, we observed lesional Th22 skewing across all body sites (Del Duca et al., 2019).

In summary, our results indicate that both the cutaneous and systemic immune responses in subjects with PN exhibit a predominant Th22/IL-22 profile. These results suggest that patients with PN may benefit from Th22/IL-22 inflammation-modulating therapies.

Limitations of this study include sample size and a predominantly African American patient study population without atopy. Consequently, our results may be limited to this specific population of patients with PN. Future studies should assess the levels of IL-22/Th22–associated genes at varying time points of PN progression. Furthermore, studies should determine immune polarization in patients with PN who are of different races and sexes.

MATERIALS AND METHODS
A prospective Johns Hopkins Institutional Review Board–approved study was performed comparing PBMCs and skin biopsies from patients with PN with those from race-, sex-, and age-matched healthy patients (Supplementary Tables S1 and S2). Written informed consent was obtained from each study participant. Body site–matched skin biopsies were collected from healthy control skin (n = 13) and pruritic lesional (n = 13) and nonpruritic, nonlesional (n = 13) skin from patients with PN. Lesional PN skin biopsy was obtained from the most pruritic nodule as self-reported by the patient. Nonlesional PN skin biopsy was obtained at least 10 cm from the PN lesional site on normal-appearing skin; healthy control skin biopsy was body site–matched with PN biopsies. Informed consent was obtained from each study participant. A schematic of the study design is shown in Supplementary Figure S11. Inclusion criteria for patients with PN consisted of clinically diagnosed PN by an experienced dermatologist and by pruritus >7 on the Itch Numeric Rating Scale (Phan et al., 2012). Patients with a known underlying or with a history of atopic or cutaneous diseases such as psoriasis or AD were excluded from the study. Adult patients with clinically determined healthy skin were included as controls and were matched to participating patients with PN by sex, race, and age.

Flow cytometry was performed using PBMCs from patients with PN (n = 5), and those from matched healthy control subjects (n = 5) were obtained (Supplementary Table S2). A total of 13 adults with histologically and clinically diagnosed PN and 13 matched control subjects (13 lesional samples, 13 nonlesional samples, and 13 healthy control samples) were included in the analysis (Supplementary Table S1). Details of H&E staining, immunofluorescence staining, flow cytometry, and mRNA sequencing are provided in the Supplementary Materials and Methods.

Analyses were performed using Excel (Microsoft, Redding, WA). Standard two-tailed t-tests were used to assess significant differences across continuous variables. In all analyses, a P-value threshold <0.05 was considered statistically significant. FACs data are presented as mean ± SEM, and violin plots are expressed with median and interquartile range. Mann–Whitney U test was performed between the healthy control samples and the PN samples. Statistics were analyzed in Prism 9.0 (GraphPad Software, San Diego, CA). Normalization and differential expression of mRNA data were carried out using the DESeq2 Bioconductor (Bioconductor) package with the R statistical programming environment (Huber et al. 2015; Love et al. 2014; The R Foundation, 2021). Paired or matched sample modeling included pair identification as a cofactor. The false discovery rate was calculated to control for multiple hypothesis testing. Immune dysregulation of gene sets was assessed using GSVAs, conducted with the GSVA R Bioconductor package using R, version 3.6.3 (Hänzelmann et al., 2013; The R Foundation, 2021). P values from paired t-tests were adjusted for multiple hypotheses using the Benjamini–Hochberg procedure (Benjamini and Hochberg, 1995).

Data availability statement
All data referenced in this study are included in the figures, tables, and Supplementary Materials and Methods.

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CONFLICT OF INTEREST
MIMK is an advisory board member and consultant for Abbvie, Galderma, Incyte Corporation, Pfizer, Regeneron Pharmaceuticals, and Kiniksa Pharmaceuticals and has received grant funding from Galderma, Pfizer, and Kiniksa Pharmaceuticals. KB and JFP are employed by Kiniksa Pharmaceuticals (Lexington, MA). The remaining authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: SGK; Formal Analysis: MB, MPA, IB, RK, TP, BH, JJ, SW, KS, YSR, NS, JC, CD, WC, DC, ND, MMK, SGK; Funding Acquisition: KB, JFP, MMK; Investigation: MB, MPA, IB, KAW, RK, BH, SW, TP, YSR, SGK; Methodology: MB, MPA, IB, JJ, CD, WC, DC, ND, MMK, SGK; Visualization: MB, IB, SW; Writing – Original Draft Preparation: MB, MPA, IB, SGK; Writing – Review and Editing: MB, MPA, IB, SW, BH, SW, YSR, NS, JC, JJ, ADJ, KS, CV, JM, KB, JFP, MMK, ALC, NKA, LAG, XD, SGK

SUPPLEMENTAL MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.02.749.

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SUPPLEMENTAL MATERIALS AND METHODS

Flow cytometry
Viable 3–5 × 10^6 cells were stimulated in RPMI with eBioscience Cell Stimulation Cocktail (plus protein transport inhibitors) (Thermo Fisher Scientific, Waltham, MA) for 4 hours at 37 °C in a 5% carbon dioxide incubator. Cells were collected after stimulation, and single-cell suspensions were obtained after filtering through a 40-μm cell filter. The cells were then washed in RPMI. The single-cell suspension was incubated with TruStain fcX (BioLegend, San Diego, CA) to block Fc receptor binding and was resuspended to label with mAbs against extracellular cell surface markers (Supplementary Table S3). The cell surface markers were incubated with the cells in Hanks Balanced Salt Solution with 2% Calf Serum and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid along with Brilliant Stain Buffer (BD Biosciences, San Jose, CA). The stained cells were washed with PBS and stained for viability (Zombie Aqua Fixable Viability Kit, BioLegend). The surface-labeled cells were fixed in the BD Cytofix/Cytoperm Buffer kit (BD Biosciences). The cells were further labeled for intracellular cytokine markers (Supplementary Table S3). The mAb-labeled cells were then washed in intracellular staining buffer and resuspended in Stabilizing Fixative (BD Biosciences). Cell acquisition was performed on the BD LSFRFortessa flow cytometer (BD Biosciences), and data were analyzed using Cytobank software (Cytobank, Mountain View, CA). The absolute number of the corresponding cell population was calculated as the total number of live cells × % of the corresponding cell population/100. For immunophenotyping of the T-cell subsets, including CD4, CD8, γδ, and invariant NK T cells, the cells were first gated on live cells, singlets and CD3+ cells, CD4+CD8− (T helper) cells, CD8+CD4− (cytotoxic T) cells, CD4+CD8−γδTCR+ (γδ T) cells, and CD4+CD8−CD56+ (invariant NK T) cells (Supplementary Figure S1).

mRNA sequencing
Patients with prurigo nodularis (PN) biopsy sites were determined through clinical presentation and the patient’s reported itch. Pruritic lesional and nonpruritic nonlesional 4-mm skin punch biopsy specimens were collected from each patient with PN. Biopsy locations of healthy controls corresponded to the biopsy site of their matched patients with PN. A total of 13 adults with histologically and clinically diagnosed PN and 13 matched control subjects were included in the analysis. The majority of the study cohort were African American (77% of total) and female (85% of total) (Supplementary Table S1).

Skin punch biopsy specimens from each participant were snap frozen in liquid nitrogen and stored at −80 °C. Extracted total RNA quality and concentration were assessed by Fragment Analyzer (Agilent Technologies, Santa Clara, CA) and Qubit 2.0 (Thermo Fisher Scientific, respectively). RNA-sequencing libraries were prepared using the commercially available KAPA Stranded mRNA-Seq Kit (Roche Holdings, Basel, Switzerland). In brief, mRNA transcripts were first captured using magnetic oligo-dT beads, fragmented using heat and magnesium, and reverse transcribed using random priming. During the second strand synthesis, the cDNA:RNA hybrid was converted into double-stranded cDNA, and deoxyribonucleoside triphosphate was incorporated into the second cDNA strand to mark the second strand. Illumina-sequencing adapters were then ligated to the double-stranded cDNA fragments and amplified to produce the final RNA-sequencing library. The strand marked with deoxyribonucleoside triphosphate was not amplified, allowing strand-specificity sequencing. Libraries were indexed using a dual indexing approach allowing for all the libraries to be pooled and sequenced on the same sequencing run. Before pooling and sequencing, fragment length distribution for each library was first assessed on a Fragment Analyzer. Libraries were also quantified using Qubit. The molarity of each library was calculated on the basis of Qubit concentration and average library size. All libraries were then pooled in equimolar ratio and sequenced. Sequencing was performed on an Illumina NovaSeq 6000 sequencer (Illumina, San Diego, CA). The pooled libraries were sequenced on an S2 flow cell at 50 base pair paired end. Once generated, sequence data were demultiplexed, and Fastq files were generated using the bc2fastq, version 2.20.0.422, file converter from Illumina.

RNA-sequencing data were processed using the fastp toolkit to trim low-quality bases and Illumina-sequencing adapters from the 3’ end of the reads (Chen et al., 2018). Only reads that were 20 nt or longer after trimming were kept for further analysis. Reads were mapped to the GRCh38v93 version of the human genome and transcriptome using the STAR RNA-seq alignment tool (Dobin et al., 2013; Kersey et al., 2012). Reads were kept for subsequent analysis if they mapped to a single genomic location. Gene counts were compiled using the feature Counts tool (Liao et al., 2014). Only genes that had at least 10 reads in any given library were used in subsequent analysis.

Gene set enrichment analysis was performed to identify gene ontology terms and the pathways associated with altered gene expression for each of the comparisons performed. In addition, to perform pathway-level comparisons between lesional, nonlesional, and healthy samples, gene set variation analyses were conducted using previously published gene sets (Dhingra et al., 2014; Gittler et al., 2012; Noda et al., 2015; Sanyal et al., 2019; Suárez-Fariñas et al., 2013). Differentially expressed genes were defined as coding genes with a log2 fold change > 1 or less than −1 and a false discovery rate–adjusted P < 0.05.

H&E and immunofluorescence
Skin punch biopsy specimens were placed in neutral-buffered 10% formalin. After fixation, specimens were embedded in paraffin and cut into slides. Slides were stained with H&E and evaluated by a blinded dermatopathologist. Matched samples were also immunofluorescence stained with antibodies for IL-1A, SERPINB4, and DAPI.

Skin punch biopsy specimens from each participant were placed in neutral-buffered 10% formalin. After fixation, specimens were embedded in paraffin and cut into slides.
and immunofluorescence triple stained with antibodies for SERPINB4 and IL-1α (ab254255 and ab7632, respectively, Abcam, Cambridge, United Kingdom) was also performed with antibodies diluted at 1:500 and 1:100. Opal immunofluorescence staining was performed according to the manufacturer’s instructions (NEL810001KT, Akoya Biosciences, Menlo Park, CA). In brief, slides were deparaffinized and hydrated in a standard histologic gradient. Three-minute Tris-buffered saline-Tween 20 washes were performed between each subsequent step. Slides were placed in a plastic chamber and retrieved in citrate buffer (H-3300, Vector Laboratories, Burlingame, CA) in a microwave, which also provided the stripping step for multiplex staining. Protein blocking (S2003, Dako, Glostrup, Denmark) was performed for 10 minutes, followed by primary antibody incubation for 45 minutes at room temperature. Slides were incubated with anti-Mouse and/or anti-Rabbit IgG (PV6119/6114, Leica, Wetzlar, Germany) secondary antibodies (as appropriate) for 30 minutes. Fluorescent stains Opal 520 (for IL-1α) and Opal 570 (for SERPINB4) were diluted in Opal amplification buffer, and slides were stained for 10 minutes. The staining process was repeated in the order of fluorescence 520, 570, then 690 nm. Slides were counterstained with DAPI working solution for 10 minutes, washed, and mounted with ProLong Gold.

**SUPPLEMENTARY REFERENCES**


Supplementary Figure S1. ×80 original magnification of H&E staining of PN lesional skin. This demonstrates a heterogeneous infiltrate of inflammatory cells, including (a) eosinophils (orange arrow), lymphocytes (blue arrow), and plasma cells (green arrow); (b) mast cells (yellow arrow) and plasma cells (green arrow); (c) eosinophils (orange arrow), lymphocytes (blue arrow), and neutrophils (black arrow); and (d) eosinophils (orange arrow) and lymphocytes (blue arrow). Bar = 40 μm. PN, prurigo nodularis.
Supplementary Figure S2. Heatmap of cutaneous gene expression with mRNA-sequencing highlights differences in immune signature in lesional PN skin. Red, greater expression; blue, lower expression. The fold change shown is log base 2 fold change. The P-value shown is a false discovery rate-adjusted P-value. *P < 0.05, **P < 0.01, ***P < 0.001.

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*P < 0.05, **P < 0.01, ***P < 0.001.
Supplementary Figure S3. Correlation of itch NRS values with GSVA scores of Th1-, Th2-, Th17-, and Th22-related genes from patients with PN and controls. Itch severity most strongly correlated with the degree of Th22-related gene expression. GSVA, gene set variation analysis; NRS, numeric rating scale; Th, T helper.
Supplementary Figure S4. Gene Set Variation Analysis of immune mediators in lesional PN skin (n = 13 samples), nonlesional skin (n = 13 samples), and matched healthy skin (n = 13 samples) by race. AAs: 10 matched patients; EAs: 3 matched patients. Th22 upregulation is observed in both EAs and AAs. *P < 0.05, **P < 0.01, and ***P < 0.001. AA, African American; EA, European American; PN, prurigo nodularis; Th, T helper.
Supplementary Figure S5. Gene Set Variation Analysis of immune mediators in lesional PN skin (n = 13), nonlesional skin (n = 13), and matched healthy skin (n = 13) by biopsy site. Arms: n = 4; legs: n = 6; and back: n = 3. Th22 upregulation is observed across all biopsy sites. *P < 0.05, **P < 0.01, and ***P < 0.001. PN, prurigo nodularis; Th, T helper.
Supplementary Figure S6. Gating strategy for cell populations with representative flow plots for the CD3⁺ T-cell population. Representative flow plots showing the gating strategy for CD8⁻ T cells (CD3⁺CD8⁻CD4⁺), CD4⁻ T cells (CD3⁺CD4⁺CD8⁻), iNK T cells (CD3⁺CD4⁻CD8⁻CD56⁺), and γδ T cells (CD3⁺CD4⁻CD8⁻γδ TCR⁺), respectively. FSC, forward scatter; FSC-A, forward scatter area; FSC-H, forward scatter height; iNK, invariant NK; SSC, side scatter; SSC-A, side scatter area; SSC-H, side scatter height.
Supplementary Figure S7. Absolute cell number for the T-cell population. Absolute cell numbers of PBMCs from patients with PN (n = 4) and healthy subjects (n = 5). (a) The absolute number of CD3^+ γδ T cells (median ± IQR). (b) The absolute number of the subpopulations of γδ T cells ± SEM. (c) The absolute number of iNK T cells (median ± IQR). (d) Representative flow cytometry plots for CD4^+ T helper (CD3^+CD4^+CD8^-) cells and CD8^+ T cytotoxic (CD3^-CD8^-CD4^-) cells. (e) Percentage of the ratio of CD4^+ and CD8^+ cell population ± SEM. (f) The absolute number of CD4^+ T cells (median ± IQR). (g) The absolute number of CD8^+ T cells (median ± IQR). (h) The absolute number of CD8-naive and CD8 memory cells (median ± IQR). *P < 0.05 for healthy controls versus subjects with PN, as calculated by a nonparametric Mann–Whitney U test. iNK, invariant NK; IQR, interquartile range; ns, nonsignificant; PN, prurigo nodularis.
Supplementary Figure S8. Absolute cell number of T-cell populations expressing cytokines. Absolute cell numbers of PBMCs from patients with PN (n = 4) and healthy subjects (n = 5) (a) Absolute number of IL-17-expressing T-cell populations ± SEM. (b) The absolute number of TNF-expressing T-cell populations ± SEM. (c) The absolute number of IL-4-expressing T-cell populations ± SEM. *P < 0.05 for healthy controls versus subjects with PN subjects, as calculated by a nonparametric Mann–Whitney U test. iNK, invariant NK; ns, nonsignificant; PN, prurigo nodularis.
Supplementary Figure S9. Intracellular cytokine profile in patients with PN. PBMCs from patients with PN (n = 4) and healthy subjects (n = 5) were stimulated with PMA and ionomycin combined with protein transport inhibitor for 4 hours. The cells were analyzed for intracellular cytokine expression by flow cytometry. (a) Percentage of TGFβ-expressing CD3+ T-cell populations ± SEM. (b) Percentage of IFN-γ-expressing CD3+ T-cell populations ± SEM. (c) Percentage of IL-17-expressing CD3+ T-cell populations ± SEM. (d) Percentage of IL-10-expressing CD3+ T-cell populations ± SEM. (e) Percentage of IL-13-expressing CD3+ T-cell populations ± SEM. *P < 0.05 for healthy controls versus subjects with PN, as calculated by a nonparametric Mann--Whitney U test. iNK, invariant NK; ns, nonsignificant; PMA, phorbol 12-myristate 13-acetate; PN, prurigo nodularis.
Representative IL-17 expression by CD3⁺ T cells. PBMCs from patients with PN (n = 4) and bacteremia (n = 5) were stimulated with PMA and ionomycin combined with protein transport inhibitor for 4 hours. The cells were analyzed for intracellular cytokine expression by flow cytometry. Representative flow cytometry histograms of intracellular IL-17 cellular expression versus that of unstained Ctrl. Ctrl, control; MFI, mean fluorescence intensity; PMA, phorbol 12-myristate 13-acetate; PN, prurigo nodularis.
Supplementary Figure S11. Outline of experimental design. Under IRB approval, PBMCs and skin samples were obtained from patients with PN and healthy subjects matched by age, race, and gender. Flow cytometric analysis compared the numbers and phenotypic characteristics of \( \gamma^\delta \^T \) cells in the peripheral blood of patients with PN with that of matched healthy controls. RNA sequencing with immunohistochemistry validation compared the gene expression in pruritic lesional PN, nonpruritic nonlesional PN, and biopsy-site–matched control skin. hrs, hours; IRB, institutional review board; PN, prurigo nodularis.
### Supplementary Table S1. Study Cohort Demographic Information for Matched Patients Recruited to Provide Skin Samples, mRNA, and Immunofluorescence Analyses

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<th>Itch NRS</th>
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Abbreviations: AA, African American; F, female; HC, healthy control; M, male; NRS, numeric rating scale; PN, prurigo nodularis.

### Supplementary Table S2. Study Cohort Demographic Information for Matched Patients Recruited to Provide PBMCS for Flow Cytometry Analyses

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Abbreviations: AA, African American; F, female; HC, healthy control; M, male; NRS, numeric rating scale; PN, prurigo nodularis.

1Too few cells were collected; therefore, patients were excluded from analyses.